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Method for preparing oligonucleotides

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### Method for preparing oligonucleotides

The present invention is related to a method for preparing oligonucleotides.

5 The synthesis of oligonucleotides has been the subject of investigations for a long period of time. Automated synthesis procedures have been developed and apparatus for the automated syntheses are commercially available. Most of these procedures have been developed for rather small quantities of oligonucleotides (in the range of mg). These amounts are sufficient for most investigational purposes.

10 Especially with the development of antisense therapeutics, large scale synthesis became a matter of considerable importance. Although relative large scale amounts of oligonucleotides have been obtained by scale-up of solid phase synthesis procedures, these technologies show major limitations especially high costs for reagents and materials, e.g. the solid phase bound starting oligonucleotide.

15 With scale-up, the reaction time of each step of the synthesis increases.

Furthermore oligonucleotides synthesis by standard solid phase synthesis results in a contamination of the desired full length compound by failure sequences arising from incomplete reaction during the synthesis cycle. At large scales the purification of the crude oligonucleotide involves complicated isolation and chromatographic purification of the final product.

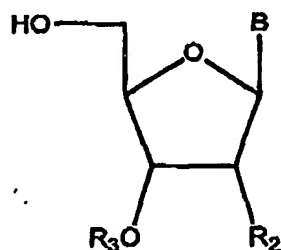
20 In general synthesis methods for oligonucleotides consist of a four-step procedure for the elongation of the oligonucleotide

1. Coupling
2. Capping
- 25 3. Oxidation
4. Deprotection of the protected hydroxyl group for the next reaction cycle.

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The object of the present invention is to provide a method for the preparation of oligonucleotides suitable for large scale (kilogram to tons) synthesis. In one embodiment this object is solved by a liquid phase synthesis method, comprising the steps of

- 5 a) providing a 3'-protected compound having the formula:



(Formula I)

wherein

B is a heterocyclic base

- 10 R<sub>2</sub> is H, a protected 2'-hydroxyl group, F, a protected amino group, an O-alkyl group, an O-substituted alkyl, a substituted alkylamino or a C4'-O2' methylen linkage

R<sub>3</sub> is a hydroxyl protecting group, a 3'-protected nucleotide or a 3'-protected oligonucleotide

- 15 b) reacting said compound with a nucleotide derivative having a 5'-protection group in the presence of a solid supported activator to give an elongated oligonucleotide with a P(III)-internucleotide bond

c) processing the elongated oligonucleotide with a P(III)-internucleotide bond by steps c1) and c2) in any sequence

- 20 c1) capping by reacting with a solid supported capping agent

c2) oxidizing by reacting the oligonucleotide with a solid supported oxidizing reagent

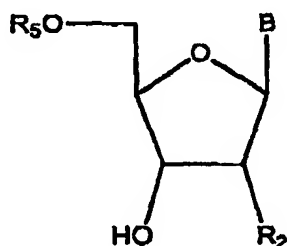
- 3 -

d) removing the 5'-protection group by treatment with a solid supported agent or removing the 5'-protection group with a removal agent followed by addition of a solid supported scavenger or followed by extraction.

The method of the present invention is a solution phase synthesis wherein the reagents are solid supported. Solid supported shall cover covalently bound reagents and reagents bound to a solid support by ionic forces.

In most cases coupling occurs of a 5'-OH-synthon with a 3'-phosphorous-synthon. Alternatively coupling of a 5'-phosphorous-synthon with a 3'-OH-synthon is also possible. Therefore in a further embodiment the invention comprises a method comprising the steps of

a) providing a 5'-protected compound having the formula:



(Formula II)

wherein

B is a heterocyclic base

R<sub>2</sub> is H, a protected 2'-hydroxyl group, F, a protected amino group, an O-alkyl group, an O-substituted alkyl, a substituted alkylamino or a C4'-O2' methylen linkage

R<sub>5</sub> is a hydroxyl protecting group, a 5'-protected nucleotide or a 5'-protected oligonucleotide

b) reacting said compound with a nucleotide derivative having a 3'-protection group in the presence of a solid supported activator to give an elongated oligonucleotide with a P(III)-internucleotide bond

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c) processing the elongated oligonucleotide with a P(III)-Internucleotide bond by  
~~steps c1) and c2) in any sequence~~

c1) capping by reacting with a solid supported activated capping agent

c2) oxidizing by reacting the oligonucleotide with a solid supported oxidizing  
5 reagent

d) removing the 3'-protection group by treatment with a solid supported agent  
or removing the 3'-protection group with a removal agent followed by addi-  
tion of a solid supported scavenger or followed by extraction.

10 In further embodiments, it is possible to couple a 3'-phosphorous synthon with  
a 3'-OH synthon to form a non-natural 3'-3'-internucleosidic linkage. For the  
synthesis of non-natural 5'-5'-internucleosidic linkages it is possible to react a  
5'-phosphorous synthon with a 5'-OH synthon. These non-natural internucleo-  
sidic linkages show increased nuclease resistance.

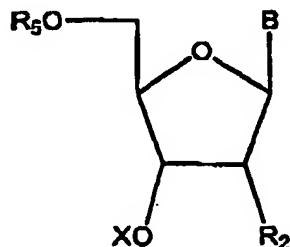
#### Step a)

15 B, the heterocyclic base can be a natural nucleobase or a modified one including  
a non-base residue. The natural nucleobases are adenine, guanine, thymine,  
cytosine and uracil. In general these bases need protection groups during the  
synthesis. Suitable protected nucleobases are known to person skilled in the art  
for example N-4-benzoylcytosine, N-6-benzoyl adenine, N-2-isobutiryl guanine,  
20 N-4-acetyl or isobutiryl cytosine, N-6-phenoxyacetyl adenine, N-2-tert-butyl  
phenoxyacetyl guanine. Suitable non-base residues include for example Hydro-  
gen, H leading to the 1',2'-dideoxyribose (dSpacer from Glen Research) which  
can be used as linker or to mimic abasic sites in an oligonucleotide (Takeshita et  
al., J. Biol. Chem., 1987, 262, 10171).

25 A suitable protection for the 2'-hydroxyl-group include but are not limited to  
tert-butyl dimethylsilyl (TBDMS), triisopropylsilyloxymethyl (TOM), fluorophenyl-  
metoxypiperidinyl (FPMP).

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Suitable protecting groups for the 3'-hydroxyl-group include but are not limited to tert-butyl dimethylsilyl (TBDMS), levulinyl, benzoyle. This compound is then reacted with a nucleotide derivative with a 3'-phosphorous-synthon. The nucleotide derivative preferably has the following formula



(Formula III)

wherein

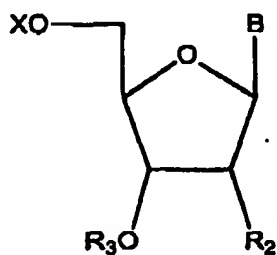
X is a P(III)-function

B is a heterocyclic base

10 R<sub>2</sub> is H, a protected 2'-hydroxyl group, F, a protected amino group, an O-alkyl group, an O-substituted alkyl, a substituted alkylamino or a C4'-O2' methylen linkage

R<sub>5</sub> is a hydroxyl protecting group, a 5'-protected nucleotide or a 5'-protected oligonucleotide.

15 In the second embodiment, the nucleotide derivative preferably has the following formula



(Formula IV)

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wherein

X is a P(III)-function

B is a heterocyclic base

5 R<sub>2</sub> is H, a protected 2'-hydroxyl group, F, a protected amino group, an O-alkyl group, an O-substituted alkyl, a substituted alkylamino or a C4'-O2'-methylen linkage

R<sub>5</sub> is a hydroxyl protecting group, a 5'-protected nucleotide or a 5'-protected oligonucleotide.

**Step b): The coupling step**

10 In step b) the coupling of the nucleotide or oligonucleotides occurs. The chemistry of the reaction depends on the type of activated phosphorous compound. Several methods for coupling nucleotides are known. The most common methods are phosphoramidite and H-phosphonate. In each of these cases the phosphorus is in an activated state which allows coupling with the free hydroxyl group  
15 of the other part.

In phosphoramidite chemistry (Beaucage et al., Tetrahedron, 1992, 48, 2223-2311; Beaucage and Caruthers in unit 3:3 of Current Protocols in Nucleic Acid Chemistry, Wiley) a nucleoside or oligonucleotide-3'-O-phosphoramidite where the P(III) phosphorus is substituted with a dialkylamine (phosphite activating  
20 group) and a phosphorus protecting group (including but not limited to 2-cyanoethyl, methyl) is reacted with 5'-hydroxyl nucleoside or oligonucleotide in presence of an activator to create a phosphite triester internucleosidic linkage.

In H-phosphonate chemistry (Froehler, Methods in Molecular Biology. Protocols for oligonucleotides and analogs, Humana Press, 1993, 63-80; Strömberg and  
25 Stawinski, In unit 3.4 of Current Protocols in Nucleic Acid Chemistry, Wiley) a nucleoside or oligonucleotide-3'-O-H-phosphonate is reacted with a 5'-hydroxyl nucleoside or oligonucleotide in presence of an activator to create a H-phosphonate diester internucleosidic linkage.

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Suitable activators for the coupling step in phosphoramidite chemistry is a solid support bearing a pyridinium salt, for example a solid support covalently linked to pyridine e.g. poly(vinyl)-pyridinium or the pyridinium is a counter ion of a cation exchange solid support. The cation exchange support can be a strong or a weak exchanger, for example a sulfonic acid or a carboxylic acid. The pyridinium salt can also be a substituted pyridinium salt, for example dichloropyridinium. It can further be a solid support bearing an optionally substituted azole (imidazole, triazole, tetrazole), or is the salt of a weak base anion exchange resin with a strong acid, or a weak cation exchange resin (carboxylic) in its protonated form (see US patent 5,574,146), or a solid support bearing an optionally substituted phenol (see W. Dabkowski and al., Tet Lett, 2000, 41, 7535-7539).

For the H-phosphonate method the suitable activators are solid supports bearing a carboxylic acid chloride, sulfonic acid chloride, a chloroformate, a chlorosulfite or a phosphorochloridate or the respective Br-compounds. Further compounds are disclosed in WO 01/64702 A1, page 6, line 36 to page 8, line 5, incorporated by reference and CB reese and Q song, Nucleic Acid Res., 1999, 27, 963-971.

#### **Step c) Capping and Oxidising**

Capping is understood as a reaction wherein a reagent reacts with remaining protected compounds of step a). As the capping agent is solid supported, the 3'-protected compound can be removed together with the solid supported capping agent.

For the capping step suitable reagents as capping agents are activated acids for example carboxylic acid, chloride or sulfonic acid chloride, carboxylic acid bromide, azolide, substituted azolide, anhydride or chloroformate or phosphorochloridate, or a solid supported phosphoramidate, or a solid supported H-phosphonate monoester. The acid group is preferably an acid group covalently bound to a solid support commercially available cationic exchanger resins can be used as a starting material for synthesizing the solid supported carboxylic acids or sulfonic acids.



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The oxidizing reaction is used to oxidize the P(III)-internucleotide bond to a P(V)-internucleotide bond. Capping can be performed before oxidizing and vice versa. Depending on the reagents capping and oxidizing may also be combined in one step.

- 5 For the oxidizing step the oxidizing reagent can be any oxidizing reagent used for prior art solid phases, but in the form of solid supported agent, either covalently bound or bound by ionic forces. Suitable reagents are solid supported periodates, permanganates, osmium tetroxides, dichromates, hydroperoxides, substituted alkylamine oxides, percarboxylic acid and persulfonic acid.
- 10 These compounds are negatively charged, therefore they can be solid supported by a suitable ion exchanger for example an ion exchanger bearing ammonium groups. These substances could be bound to solid support consisting for example of an amino, alkyl amino, dialkyl amino or trialkyl amino anion exchanger.

- In oligonucleotides synthesis for investigational purposes and especially for antisense therapeutics phosphorothioate analogs are used. In this case the oxidizing is a sulfurization. As a solid supported oxidizing reagent a solid supported sulfurization reagent is used, for example a solid supported tetrathionate, a solid supported alkyl or aryl sulfonyl disulfide, a solid supported optionally substituted dibenzoyl tetrasulfide, a solid supported bis(alkoxythiocarbonyl)tetrasulfide, a solid supported optionally substituted phenylacetyl disulfide, a solid supported N-[(alkyl or aryl)sulfanyl] alkyl or aryl substituted succinimide and a solid supported (2-pyridinyldithio) alkyl or aryl.
- 15
- 20

#### **Step d) Deprotection**

- As a 5'-protection group suitable groups are trityl groups, preferably a dimethoxytrityl group (DMTr) or a monomethoxytrityl group (MMTr). These protection groups are used in conventional prior art solid phase oligonucleotides synthesis. Other suitable 5'-protection groups are included but are not limited to tert-butyl dimethylsilyl (TBDMS), levulinyl, benzoyl, fluorenylmethoxycarbonyl (Fmoc), the 9-phenylthioxanthen-9-yl (S-pixyl).
- 25

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In the second embodiment, in step d) the 3'-protection group is removed. Suitable 3'-protection groups are 3'-O-*tert* butyl dimethyl silyl (TBDMS), 3'-O-acetate, 3'-O-levulinyl groups. They can be removed by a solid-supported ammonium fluoride, solid-supported ammonium hydroxide or solid-supported hydrazine.

In step d) of the first embodiment, the 5'-protection group is removed. Thereafter the oligonucleotides can either be used or the oligonucleotide corresponds to the 3'-protected compound of step a) to repeat the cycle.

The use of solid supported reagents for the removal of the DMTr-protection group for a completely synthesized oligonucleotide has already been reported in US 5,808,042. The content of this document is incorporated by reference. Surprisingly the methods disclosed in US 5,808,042 can also be applied in a solution phase synthesis as described in the present application.

In step d) of the second embodiment, the 3'-protection group is removed. Thereafter the oligonucleotides can either be used or the oligonucleotide corresponds to the 3'-protected compound of step a) to repeat the cycle.

#### **Step e): Repetition**

In most cases the methods will be repeated at least once. When starting from monomeric oligonucleotides the method of the present invention will result in a dimer. Repeating the method of the present invention will elongate the dimer to a trimer. By repeating the method of the invention several times n-mers can be synthesized.

As the yield of a synthesis is not 100%, the overall yield of correct oligonucleotides decreases with the number of cycles. Depending on the yield of a single cycle, oligonucleotides can be synthesized of up to 100 nucleotides in sufficient yield. Longer oligonucleotides are also possible.

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For most cases oligonucleotides having that size will not be needed. An antisense therapy oligonucleotides are normally in the range of 8-36 nucleotides, more preferably 12-30, most commonly in the range of the 16-26 nucleotides.

In contrast to prior art, convergent synthesis strategies are fully compatible with the synthesis method of the present invention. Convergent synthesis methods are methods wherein small oligonucleotides are synthesized first and the small oligonucleotides are then combined for synthesizing larger blocks. By this method the number of coupling reactions can be significantly reduced. Thereby the overall yield of the oligonucleotide is increased.

10 In prior art, each synthesis of a small oligonucleotide had to start from the solid support bound nucleic acid which was rather expensive. Therefore convergent synthesis strategies have not found much application in oligonucleotide synthesis.

15 Convergent synthesis has the further advantage, that the reaction product is essentially free of (n-1)mers. In prior art synthesis, the purification of oligonucleotides with a length of n from oligonucleotides with a length of n-1 is the most difficult in purification of the oligonucleotide. By convergent synthesis, these (n-1)mers are nearly avoided, because larger fragments are combined.

20 In a preferred embodiment, the method of the present invention uses dimers or trimers as the compounds in step a and/or b.

During the synthesis cycles, reagents are added in an solid supported form. These solid supported reagents are preferably removed after reaction after each reaction step. Depending on the type of reagent it is in some cases possible to remove two or more of the solid supported reagents together.

25 As the synthesis is intended for the production of large amounts of oligonucleotides it is preferred that the solid supported reagent is recycled. This recycling is obviously easier if the solid supported reagents are removed separately after each reaction.

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The solid supported reagents can be removed by methods like filtration or centrifugation. Because of the ease of handling, filtration is the preferred way of removing the solid supported reagents.

5 A very preferred reagent for the sulfurization is a solid supported anion exchange resin in complex with a tetrathionate having the formula  $S_4O_6$ , preferably a quaternary ammonium resin bearing tetrathionate as counter ion.

The invention will be further exemplified with the following examples.

### **Example 1**

10 *Synthesis of the dimer 5'-O-DMTr-T-T-3'-O-TBDMS cyanoethyl phosphite triester.*

**Coupling procedure** of 5'-O-DMTr-T-3'-cyanoethyl phosphoramidite with 5'-OH-T-3'-O-TBDMS using the DOWEX 50W X8 pyridinium form.

### **Analytical scale.**

15 5'-OH-T-3'-O-TBDMS (11 mg, 32.5 mmol) and 5'-O-DMTr-T-3'-cyanoethyl phosphoramidite (41 mg, 55.25 mmol, 1.7 eq) are dissolved in anhydrous acetonitrile (550 ml). The solution is transferred under argon in a NMR tube containing the DOWEX 50W X8 pyridinium form (100 mg, 0.30 mmol  $pyrH^+$ , 9.2 eq). The reaction is followed by  $^{31}P$  NMR. Before the NMR experiment deuterated acetonitrile (50 ml) is added. The yield is determined by  $^{31}P$  NMR.

20 After 3 h the desired dimer T-T phosphite triester is obtained with 100% of yield compared to 5'-OH-T-3'-O-TBDMS. The crude is a mixture of 5'-O-DMTr-T-3'-cyanoethyl phosphoramidite ( $^{31}P$  NMR ( $CD_3CN$ )  $\delta$  149.14, 149.07, 14.7%), 5'-O-DMTr-T-T-3'-O-TBDMS cyanoethyl phosphite triester ( $\delta$  140.53, 70.2%), 5'-O-DMTr-T-3'-cyanoethyl hydrogenophosphonate ( $\delta$  8.74, 15.1%).

### 25 **Example 2**

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*Synthesis of the dimer 5'-O-DMTr-T-T-3'-O-TBDMS cyanoethyl phosphite triester.*

**Coupling procedure** of 5'-O-DMTr-T-3'-cyanoethyl phosphoramidite with 5'-OH-T-3'-O-TBDMS using the poly(4-vinylpyridinium *p*-toluenesulfonate) (Aldrich).

**Analytical scale.**

5'-OH-T-3'-O-TBDMS (11 mg, 32.5 mmol) and 5'-O-DMTr-T-3'-cyanoethyl phosphoramidite (41 mg, 55.25 mmol, 1.7 eq) are dissolved in anhydrous acetonitrile (550 ml). The solution is transferred under argon in a NMR tube containing the poly(4-vinylpyridinium *p*-toluenesulfonate) (100 mg, 0.33 mmol  $\text{tos}^-$ , 10.3 eq). The reaction is followed by  $^{31}\text{P}$  NMR. Before the NMR experiment deuterated acetonitrile (50 ml) is added. The yield is determined by  $^{31}\text{P}$  NMR. After 1 h 45 the desired dimer T-T phosphite triester is obtained with 82% of yield compared to 5'-OH-T-3'-O-TBDMS. The crude is a mixture of 5'-O-DMTr-T-T-3'-O-TBDMS cyanoethyl phosphite triester ( $^{31}\text{P}$  NMR ( $\text{CD}_3\text{CN}$ )  $\delta$  140.54, 48.2%), 5'-O-DMTr-T-3'-cyanoethyl hydrogenophosphonate ( $\delta$  8.77, 51.8%).

**Example 3**

*Synthesis of the dimer 5'-OH-T-T-3'-O-TBDMS cyanoethyl phosphorothioate triester.*

**Coupling procedure** of 5'-O-DMTr-T-3'-cyanoethyl phosphoramidite with 5'-OH-T-3'-O-TBDMS using the DOWEX 50W X8 pyridinium form.

A solution of 5'-OH-T-3'-O-TBDMS (124 mg, 0.35 mmol) and 5'-O-DMTr-T-3'-cyanoethyl phosphoramidite (441 mg, 0.59 mmol, 1.7 eq) in anhydrous acetonitrile (6 ml) is added to DOWEX 50W X8 pyridinium form (1 g, 3 mmol  $\text{pyrH}^+$ , 9.5 eq). The resulting mixture is shaken for 4 h 45. The reaction is followed by  $^{31}\text{P}$  NMR and the yield is also determined by  $^{31}\text{P}$  NMR. The desired dimer 5'-O-

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DMTr-T-T-3'-O-TBDMS cyanoethyl phosphite triester is obtained with 100% of yield compared to 5'-OH-T-3'-O-TBDMS. The crude is a mixture of 5'-O-DMTr-T-3'-cyanoethyl phosphoramidite ( $^{31}\text{P}$  NMR ( $\text{CD}_3\text{CN}$ )  $\delta$  149.17, 149.10, 5.4%), 5'-O-DMTr-T-T-3'-O-TBDMS cyanoethyl phosphite triester ( $\delta$  140.57, 140.54, 68.3%), 5'-O-DMTr-T-3'-cyanoethyl hydrogenophosphonate ( $\delta$  8.75, 8.71, 26.3%).

**Sulfurization:** The DOWEX 50W X8 resin is filtered off and the resulting solution is added to AMBERLYST A26 tetrathionate form (1.44 g, 2.44 mmol  $\text{S}_4\text{O}_6^{2-}$ , 7 eq.). The reaction is followed by  $^{31}\text{P}$  NMR and the yield is also determined by  $^{31}\text{P}$  NMR. After 20 h the desired dimer 5'-O-DMTr-T-T-3'-O-TBDMS cyanoethyl phosphorothioate triester is obtained with 97% of yield. The crude is a mixture of 5'-O-DMTr-T-3'- cyanoethyl thiophosphoramidate ( $^{31}\text{P}$  NMR ( $\text{CD}_3\text{CN}$ )  $\delta$  71.16, 4.0%), 5'-O-DMTr-T-T-3'-O-TBDMS cyanoethyl phosphorothioate triester ( $\delta$  68.28, 68.23, 69.5%), 5'-O-DMTr-T-3'-cyanoethyl hydrogenophosphonate ( $\delta$  8.75, 8.71, 26.5%). MALDI-TOF MS (negative mode, trihydroxyacetophenone as matrix) ammonia treatment of an aliquot gives 5'-OH-T-T- 3'-O-TBDMS phosphorothioate diester:  $[\text{M}-\text{H}]^-$   $m/z_{\text{exp}} = 978.12$ ,  $m/z_{\text{calc}} = 977.13$ .

**Detritylation:** The AMBERLYST A26 is filtered off and the solvent are evaporated. The crude is dissolved in 4ml of  $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$  (7/3) and cooled in an ice bath. To this solution is added 1 ml of a solution of benzene sulfonic acid 10% in  $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$  (7/3). The solution is stirred 15 min at  $0^\circ\text{C}$ . The reaction is washed with 10 ml of a saturated solution of  $\text{NaHCO}_3$ , the organic layer is separated, dried ( $\text{Na}_2\text{SO}_4$ ), evaporated, and purified on a silica gel column. The desired dimer T-T is eluted with  $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$  (95/5). The appropriate fractions are collected and evaporated to give 230 mg of a white foam in a yield of 83% compared to 5'-OH-T-3'-O-TBDMS.  $^{31}\text{P}$  NMR ( $\text{CD}_3\text{CN}$ )  $\delta$  68.29, 68.19. MALDI-TOF MS (positive mode, trihydroxyacetophenone as matrix)  $[\text{M}+\text{H}]^+$   $m/z_{\text{exp}} = 730.46$ ,  $m/z_{\text{calc}} = 730.82$ . The spectrophotometric purity (97%) is determined by HPLC at 260 nm.

#### 30 Example 4

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*Synthesis of the trimer 5'-OH-T-T-T-3'-O-TBDMS cyanoethyl phosphorothioate triester.*

**Coupling procedure** of 5'-O-DMTr-T-3'-cyanoethyl phosphoramidite with the dimer 5'-OH-T-T-3'-O-TBDMS cyanoethyl phosphorothioate triester using the  
5 DOWEX 50W X8 pyridinium form.

A solution of 5'-OH-T-T-3'-O-TBDMS cyanoethyl phosphorothioate triester (230 mg, 0.31 mmol) and 5'-O-DMTr-T-3'-cyanoethyl phosphoramidite (399 mg, 0.54 mmol, 1.7 eq) in anhydrous acetonitrile (8 ml) is added to DOWEX 50W X8 pyridinium form (1 g, 3 mmol pyrH<sup>+</sup>, 9.5 eq). The resulting mixture is  
10 shaken for 5 h. The reaction is followed by <sup>31</sup>P NMR and the yield is also determined by <sup>31</sup>P NMR. The desired trimer 5'-O-DMTr-T-T-T-3'-O-TBDMS cyanoethyl phosphite triester is obtained with 100% of yield compared to the dimer 5'-OH-T-T-3'-O-TBDMS cyanoethyl phosphorothioate triester. The crude is a mixture of 5'-O-DMTr-T-3'-cyanoethyl phosphoramidite (<sup>31</sup>P NMR (CD<sub>3</sub>CN) d  
15 149.16, 149.10, 17.7%), 5'-O-DMTr-T-T-T-3'-O-TBDMS cyanoethyl phosphite triester (d 140.85, 140.68, 140.37, 140.30, d 68.07, 68.02, 68.3%), 5'-O-DMTr-T-3'-cyanoethyl hydrogenophosphonate (d 8.7, 8.68, 14%).

**Sulfurization:** The DOWEX 50W X8 pyridinium form is filtered off and the resulting solution is added to AMBERLYST A26 tetrathionate form (1.3 g, 2.44  
20 mmol S<sub>4</sub>O<sub>6</sub><sup>2-</sup>, 7 eq.). The reaction is followed by <sup>31</sup>P NMR and the yield is also determined by <sup>31</sup>P NMR. After 45 h the desired trimer 5'-O-DMTr-T-T-T-3'-O-TBDMS cyanoethyl phosphorothioate triester is obtained with 100% of yield. MALDI-TOF MS (negative mode, trihydroxyacetophenone as matrix) [M-H]<sup>-</sup> m/z<sub>exp</sub> = 1297.89, m/z<sub>calc</sub> = 1296.38 after 30 min of ammonia treatment to  
25 remove the cyanoethyl protecting group. The crude is a mixture of 5'-O-DMTr-T-3'-cyanoethyl thiophosphoramidate (<sup>31</sup>P NMR (CD<sub>3</sub>CN) d 72.04, 71.17, 14.0%), 5'-O-DMTr-T-T-T-3'-O-TBDMS cyanoethyl phosphorothioate triester (d 68.17, 68.12, 68.07, 67.96, 67.80, 67.58, 73.8%), 5'-O-DMTr-T-3'-cyanoethyl hydrogenophosphonate (d 8.76, 8.71, 12.2%).

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**Detritylation:** The AMBERLYST A26 is filtered off and the solvent are evaporated. The crude is dissolved in 4ml of  $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$  (7/3) and cooled in an ice bath. To this solution is added 1 ml of a solution of benzene sulfonic acid 10% in  $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$  (7/3). The solution is stirred 45 min at  $0^\circ\text{C}$ . The reaction is washed with 10 ml of a saturated solution of  $\text{NaHCO}_3$ , the organic layer is separated, dried ( $\text{Na}_2\text{SO}_4$ ), evaporated, and purified on a silica gel column. The desired trimer T-T-T is eluted with  $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$  (95/5). The appropriate fractions are collected and evaporated to give 221 mg of a white foam in a yield of 63% compared to the dimer 5'-OH-T-T-3'-O-TBDMS cyanoethyl phosphorothioate triester.  $^{31}\text{P}$  NMR ( $\text{CD}_3\text{CN}$ )  $\delta$  68.53, 68.38, 68.34, 67.74, 67.54. MALDI-TOF MS (positive mode, trihydroxyacetophenone as matrix)  $[\text{M}+\text{H}]^+$   $m/z_{\text{exp}} = 1103.91$ ,  $m/z_{\text{calc}} = 1104.15$ . The spectrophotometric purity (93%) is determined by HPLC at 260 nm.

### Example 5

- 15 *Synthesis of the dimer 5'-OH-T-dA<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphorothioate triester.*

**Coupling procedure** of 5'-O-DMTr-T-3'-phosphoramidite with 5'-OH-dA<sup>Bz</sup>-3'-O-TBDMS using the poly(4-vinylpyridinium *p*-toluenesulfonate) (Aldrich).

- A solution of 5'-OH-dA<sup>Bz</sup>-3'-O-TBDMS (176 mg, 0.38 mmol) and 5'-O-DMTr-T-3'-cyanoethyl phosphoramidite (560 mg, 0.75 mmol, 2 eq) in anhydrous acetonitrile (6 ml) is added to poly(4-vinylpyridinium *p*-toluenesulfonate) (1.15 g, 3.84 mmol  $\text{tos}^-$ , 10.2 eq). The resulting mixture is shaken for 4 h 30 min. The reaction is followed by  $^{31}\text{P}$  NMR and the yield is also determined by  $^{31}\text{P}$  NMR. The desired dimer 5'-O-DMTr-T-dA<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphite triester is obtained with 100 % of yield compared to the 5'-OH-dA<sup>Bz</sup>-3'-O-TBDMS. The crude is a mixture of 5'-O-DMTr-T-3'-cyanoethyl phosphoramidite ( $^{31}\text{P}$  NMR ( $\text{CD}_3\text{CN}$ )  $\delta$  149.10, 149.05, 12.3%), 5'-O-DMTr-T-dA<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphite triester ( $\delta$  140.52, 140.37, 50%), 5'-O-DMTr-T-3'-cyanoethyl hydrogenophosphonate ( $\delta$  8.72, 8.69, 37.7%).



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**Sulfurization:** The poly(4-vinylpyridinium *p*-toluenesulfonate) is filtered off and the resulting solution is added to AMBERLYST A26 tetrathionate form (1.55 g, 2.63 mmol  $S_4O_6^{2-}$ , 7 eq.). The reaction is followed by  $^{31}P$  NMR. The reaction mixture is shaken for 24 h 30. The desired dimer 5'-O-DMTr-T-dA<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphorothioate triester is isolated after filtration of the resin, evaporation of the solvent, and column chromatography (silica gel;  $CH_2Cl_2$  / MeOH (50/1)). Yield : 325 mg, 0.28 mmol, 76%.  $^{31}P$  NMR ( $CD_3CN$ )  $\delta$  68.34, 68.15. MALDI-TOF MS (positive mode, trihydroxyacetophenone as matrix)  $[M+H]^+$   $m/z_{exp} = 1144.22$ ,  $m/z_{calc} = 1146.32$ .

**Detritylation:** The 5'-O-DMTr-T-dA<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphorothioate triester is dissolved in 10 ml of  $CH_2Cl_2/CH_3OH$  (7/3) and cooled in an ice bath. To this solution is added 1 ml of a solution of benzene sulfonic acid 10% in  $CH_2Cl_2/CH_3OH$  (7/3). The solution is stirred 35 min at 0°C. The reaction is washed with 20 ml of a saturated solution of  $NaHCO_3$ , the organic layer is separated, dried ( $Na_2SO_4$ ), evaporated, and purified on a silica gel column. The desired dimer T-dA<sup>Bz</sup> is eluted with  $CH_2Cl_2/CH_3OH$  (95/5). The appropriate fractions are collected and evaporated to give a white foam. Yield: 223 mg, 0.26 mmol, 71%.  $^{31}P$  NMR ( $CD_3CN$ )  $\delta$  68.06, 67.89. MALDI-TOF MS (positive mode, trihydroxyacetophenone as matrix)  $[M-H]^+$   $m/z_{exp} = 842.18$ ,  $m/z_{calc} = 843.95$ ; (negative mode, trihydroxyacetophenone as matrix) ammonia treatment of an aliquot gives 5'-OH-T-dA-3'-O-TBDMS phosphorothioate diester:  $[M-H]^-$   $m/z_{exp} = 685.38$ ,  $m/z_{calc} = 684.77$ .

### Example 6

*Synthesis of the trimer 5'-O-DMTr-dA<sup>Bz</sup>-T-dA<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphorothioate triester.*

**Coupling procedure** of 5'-O-DMTr-dA<sup>Bz</sup>-3'-cyanoethyl phosphoramidite with the dimer 5'-OH-T-dA<sup>Bz</sup>-3'-O-TBDMS phosphorothioate triester using the poly(4-vinylpyridinium *p*-toluenesulfonate) (Aldrich).

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A solution of the dimer 5'-OH-T-dA<sup>Bz</sup>-3'-O-TBDMS phosphorothioate triester (223 mg, 0.26 mmol) and 5'-O-DMTr-dA<sup>Bz</sup>-3'-cyanoethyl phosphoramidite (432 mg, 0.50 mmol, 1.9 eq) in anhydrous acetonitrile (20 ml) is added to poly(4-vinylpyridinum *p*-toluenesulfonate) (0.8 g, 2.7 mmol tos<sup>-</sup>, 10.3 eq). The resulting mixture is shaken for 6 h 30. The reaction is followed by <sup>31</sup>P NMR and the yield is also determined by <sup>31</sup>P NMR. The desired trimer 5'-O-DMTr-dA<sup>Bz</sup>-T-dA<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphite triester is obtained with 62% of yield compared to the dimer 5'-OH-T-dA<sup>Bz</sup>-3'-O-TBDMS phosphorothioate triester. The crude is a mixture of 5'-O-DMTr-dA<sup>Bz</sup>-3'-cyanoethyl phosphoramidite (<sup>31</sup>P NMR (CD<sub>3</sub>CN) d 149.14, 8.4%), 5'-O-DMTr-dA<sup>Bz</sup>-T-dA<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphite triester (d 140.90, 140.77, 67.85, 67.79, 43.3%), 5'-OH-T-dA<sup>Bz</sup>-3'-O-TBDMS phosphorothioate triester (d 68.03, 67.89, 13.4%), 5'-O-DMTr-dA<sup>Bz</sup>-3'-cyanoethyl hydrogenophosphonate (d 8.71, 8.66, 34.9%).

**Sulfurization:** The poly(4-vinylpyridinum *p*-toluenesulfonate) is filtered off and the resulting solution is added to AMBERLYST A26 tetrathionate form (0.78 g, 1.33 mmol S<sub>4</sub>O<sub>6</sub><sup>2-</sup>, 5 eq.). The reaction is followed by <sup>31</sup>P NMR and the yield is also determined by <sup>31</sup>P NMR. After 14 h 30 the desired trimer 5'-O-DMTr-dA<sup>Bz</sup>-T-dA<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphorothioate triester is obtained with 100% of yield. The crude is a mixture of 5'-O-DMTr-dA<sup>Bz</sup>-3'-cyanoethyl thiophosphoramidate (<sup>31</sup>P NMR (CD<sub>3</sub>CN) d 71.88, 71.21, 10%), 5'-O-DMTr-dA<sup>Bz</sup>-T-dA<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphorothioate triester (25.9%) and 5'-OH-T-dA<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphorothioate triester (16.2%) (d 68.08, 68.05, 67.93, 67.89, 67.85, 67.79, 67.57), 5'-O-DMTr-T-3'-cyanoethyl phosphorothioate diester (d 57.38, 4.8%), 5'-O-DMTr-dA<sup>Bz</sup>-3'-cyanoethyl hydrogenophosphonate (d 8.75, 8.70, 43.11%). MALDI-TOF MS (positive mode, trihydroxyacetophenone as matrix) [M+H]<sup>+</sup> m/z<sub>exp</sub> = 1631.68, m/z<sub>calc</sub> = 1632.77; (negative mode, trihydroxyacetophenone as matrix) ammonia treatment of an aliquot gives 5'-OH-T-dA-3'-O-TBDMS phosphorothioate diester: [M-H]<sup>-</sup> m/z<sub>exp</sub> = 1316.45, m/z<sub>calc</sub> = 1316.43.

### Example 7

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*Synthesis of the dimer 5'-O-DMTr-dC<sup>Bz</sup>-T-3'-O-Lev cyanoethyl phosphite triester.*

**Coupling procedure** of 5'-O-DMTr-dC<sup>Bz</sup>-3'-cyanoethyl phosphoramidite with 5'-OH-T-3'-O-Lev using the DOWEX 50W X8 pyridinium form.

**5 Analytical scale.**

5'-OH-T-3'-O-Lev (20 mg, 58.9  $\mu$ mol) and 5'-O-DMTr-dC<sup>Bz</sup>-3'-cyanoethyl phosphoramidite (83.4 mg, 100  $\mu$ mol, 1.7 eq) are dissolved in anhydrous acetonitrile (550 ml). The solution is transferred under argon in a NMR tube containing the DOWEX 50W X8 pyridinium form (181 mg, 0.54 mmol pyrH<sup>+</sup>, 9.2 eq). The reaction is followed by <sup>31</sup>P NMR. Before the NMR experiment deuterated acetonitrile (50 ml) is added. The yield is determined by <sup>31</sup>P NMR. After 6 h the desired dimer T-dC<sup>Bz</sup> phosphite triester is obtained with 100% of yield compared to 5'-OH-T-3'-O-Lev. The crude is a mixture of 5'-O-DMTr-dC<sup>Bz</sup>-3'-cyanoethyl phosphoramidite (<sup>31</sup>P NMR (CD<sub>3</sub>CN)  $\delta$  149.36, 149.32, 11%), 5'-O-DMTr-T-dC<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphite triester ( $\delta$  140.52, 140.39, 70%), 5'-O-DMTr-dC<sup>Bz</sup>-3'-cyanoethyl hydrogenophosphonate ( $\delta$  8.90, 8.58, 19%).

**Example 8**

*Synthesis of the dimer 5'-OH-dC<sup>Bz</sup>-T-3'-O-Lev cyanoethyl phosphorothioate triester.*

**Coupling procedure** of 5'-O-DMTr-dC<sup>Bz</sup>-3'-cyanoethyl phosphoramidite with 5'-OH-T-3'-O-Lev using the DOWEX 50W X8 pyridinium form.

A solution of 5'-OH-T-3'-O-Lev (119 mg, 0.35 mmol) and 5'-O-DMTr-dC<sup>Bz</sup>-3'-cyanoethyl phosphoramidite (496 mg, 0.60 mmol, 1.7 eq) in anhydrous acetonitrile (10 ml) is added to DOWEX 50W X8 pyridinium form (1.1 g, 3.3 mmol pyrH<sup>+</sup>, 9.4 eq). The resulting mixture is shaken for 5 h 30 min. The reaction is followed by <sup>31</sup>P NMR and the yield is also determined by <sup>31</sup>P NMR. The desired

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dimer 5'-O-DMTr-dC<sup>Bz</sup>-T-3'-O-Lev cyanoethyl phosphite triester is obtained with 100% of yield compared to 5'-OH-T-3'-O-Lev. The crude is a mixture of 5'-O-DMTr-T-dC<sup>Bz</sup>-3'-O-Lev cyanoethyl phosphite triester (<sup>31</sup>P NMR (CD<sub>3</sub>CN) d 140.59, 140.45, 64%), 5'-O-DMTr-dC<sup>Bz</sup>-3'-cyanoethyl hydrogenophosphonate  
5 (d 8.68, 8.66, 36%).

**Sulfurization:** The DOWEX 50W X8 resin is filtered off and the resulting solution is added to AMBERLYST A26 tetrathionate form (1.44 g, 2.44 mmol S<sub>4</sub>O<sub>6</sub><sup>2-</sup>, 7 eq.). The reaction is followed by <sup>31</sup>P NMR. The reaction mixture is shaken for 16 h. The desired dimer 5'-O-DMTr-dC<sup>Bz</sup>-T-3'-O-Lev cyanoethyl phosphorothioate triester is isolated after filtration of the resin, evaporation of the  
10 solvent and column chromatography (silica gel; CH<sub>2</sub>Cl<sub>2</sub> / MeOH (97/3)). The crude is a mixture of 5'-O-DMTr-T-dC<sup>Bz</sup>-3'-O-Lev cyanoethyl phosphorothioate triester (<sup>31</sup>P NMR (CD<sub>3</sub>CN) d 68.05, 67.89, 83.7%), 5'-O-DMTr-dC<sup>Bz</sup>-3'-cyanoethyl hydrogenophosphonate (d 8.63, 16.3%). The spectrophotometric  
15 purity determined by HPLC at 260 nm is 80%.

**Detritylation of the dimer 5'-O-DMTr-dC<sup>Bz</sup>-T-3'-O-Lev cyanoethyl phosphorothioate triester with the DOWEX 50 W X8 H<sup>+</sup> form (Aldrich).**

To the mixture of the dimer 5'-O-DMTr-dC<sup>Bz</sup>-T-3'-O-Lev cyanoethyl phosphorothioate triester (121 mmol estimated) and 5'-O-DMTr-T-3'-cyanoethyl  
20 hydrogenophosphonate diester (44 mmol estimated) in solution in 10 ml of CH<sub>2</sub>Cl<sub>2</sub>/MeOH (7/3) is added the DOWEX 50 W X8 H<sup>+</sup> form (1.4 g, 7 mmol H<sup>+</sup>, 58 eq / dimer). The reaction is followed by reverse phase HPLC. After 15 min the detritylation is complete. The resin is filtered off and the solvents are evaporated. The desired dimer 5'-OH-dC<sup>Bz</sup>-T-3'-O-Lev cyanoethyl phosphorothioate triester is purified by precipitation from CH<sub>2</sub>Cl<sub>2</sub>/MeOH (9/1) in  
25 diethylether. <sup>31</sup>P NMR (CD<sub>3</sub>OD) d 68.24, 67.90, MALDI-TOF MS (positive mode, trihydroxyacetophenone as matrix) [M-H]<sup>+</sup> m/z<sub>exp</sub> = 803.11 m/z<sub>calc</sub> = 803.76. The spectrophotometric purity (97 %) is determined by HPLC at 260 nm.

### Example 9

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*Synthesis of the dimer 5'-OH-T-T-3'-O-Lev cyanoethyl phosphorothioate triester.*

**Coupling procedure of 5'-O-DMTr-T-3'-cyanoethyl phosphoramidite with 5'-OH-T-3'-O-Lev using the DOWEX 50W X8 pyridinium form.**

- 5 A solution of 5'-OH-T-3'-O-Lev (100 mg, 0.29 mmol) and 5'-O-DMTr-T-3'-cyanoethyl phosphoramidite (547 mg, 0.73 mmol, 2.5 eq) in anhydrous acetonitrile (10 ml) is added to DOWEX 50W X8 pyridinium form (0.9 g, 2.7 mmol  $\text{pyrH}^+$ , 9.3 eq). The resulting mixture is shaken for 10 h. The reaction is followed by  $^{31}\text{P}$  NMR and by reverse phase HPLC. The excess of 5'-O-DMTr-T-3'-
- 10 cyanoethyl phosphoramidite is hydrolysed with 500 ml of water. The desired dimer 5'-O-DMTr-T-T-3'-O-Lev cyanoethyl phosphite triester is obtained with 100% of yield compared to 5'-OH-T-3'-O-Lev. The crude is a mixture of 5'-O-DMTr-T-T-3'-O-Lev cyanoethyl phosphite triester (HPLC % Area = 55%) and 5'-O-DMTr-T-3'-cyanoethyl hydrogenophosphonate (HPLC % Area = 45%) .
- 15 **Sulfurization:** The DOWEX 50W X8 resin is filtered off and the resulting solution is added to AMBERLYST A26 tetrathionate form (0.8 g, 1.5 mmol  $\text{S}_4\text{O}_6^{2-}$ , 5 eq.). The reaction is followed by  $^{31}\text{P}$  NMR and by reverse phase HPLC. After 15 h the desired dimer 5'-O-DMTr-T-T-3'-O-Lev cyanoethyl phosphorothioate triester is obtained with 100% of yield. The crude is a mixture of 5'-O-DMTr-T-
- 20 T-3'-O-Lev cyanoethyl phosphorothioate triester ( $^{31}\text{P}$  NMR d 68.04, HPLC % Area = 57%), 5'-O-DMTr-T-3'-cyanoethyl hydrogenophosphonate (d 8.77, HPLC % Area = 43%).

**Detritylation of the dimer 5'-O-DMTr-T-T-3'-O-Lev cyanoethyl phosphorothioate triester with the DOWEX 50 W X8  $\text{H}^+$  form (Aldrich).**

- 25 The AMBERLYST A26 resin is filtered off and the solvents are evaporated. To the mixture of the dimer 5'-O-DMTr-T-T-3'-O-Lev cyanoethyl phosphorothioate triester (0.29 mmol estimated) and 5'-O-DMTr-T-3'-cyanoethyl hydrogenophosphonate (0.22 mmol estimated) in solution in 20 ml of  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  (7/3) is added the DOWEX 50 W X8  $\text{H}^+$  form (3.7 g, 18.5 mmol  $\text{H}^+$ , 64 eq / dimer).

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The reaction is followed by reverse phase HPLC. After 30 min the detritylation of the dimer is complete. The resin is filtered off and the solvents are evaporated. The desired dimer 5'-OH-T-T-3'-O-Lev cyanoethyl phosphorothioate triester is purified by precipitation from CH<sub>2</sub>Cl<sub>2</sub>/MeOH (9/1) in diethylether. <sup>31</sup>P NMR (CD<sub>3</sub>CN) δ 67.88, 67.73. MALDI-TOF MS (positive mode, trihydroxyacetophenone as matrix) [M-H]<sup>+</sup> m/z<sub>exp</sub> = 713.79 m/z<sub>calc</sub> = 714.66. The purity (95%) is determined by HPLC.

**Example 10**

*Synthesis of dimer 5'-O-DMTr-dA<sup>Bz</sup>-dA<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphorothioate triester dimer.*

**Coupling procedure** of 5'-O-DMTr-dA<sup>Bz</sup>-3'-cyanoethyl-phosphoramidite with 5'-OH-dA<sup>Bz</sup>-3'-O-TBDMS using the DOWEX 50W X8 pyridinium form:

5'-OH-dA<sup>Bz</sup>-3'-O-TBDMS (100 mg, 0.21 mmol) and 5'-O-DMTr-dA<sup>Bz</sup>-3'-cyanoethyl-phosphoramidite (311 mg, 0.36 mmol, 1.7 eq) are dissolved in anhydrous acetonitrile (15 ml). The solution is transferred under argon in a flask containing the DOWEX 50W X8 pyridinium form (655 mg, 1.97 mmol pyrH<sup>+</sup>, 9.2 eq) and is shaken for 4 h 30 min. The reaction is followed by <sup>31</sup>P NMR. The yield is determined by <sup>31</sup>P NMR. The desired dA-dA phosphite triester dimer is obtained with 92% of yield compared to the 5'-OH-dA<sup>Bz</sup>-3'-O-TBDMS. The crude is a mixture of 5'-O-DMTr-dA<sup>Bz</sup>-3'-cyanoethyl phosphoramidite (<sup>31</sup>P NMR (CD<sub>3</sub>CN) δ 149.25, 149.13; 27.7%), 5'-O-DMTr-dA<sup>Bz</sup>-dA<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphite triester (δ 140.75, 140.38; 53.9%), 5'-O-DMTr-dA<sup>Bz</sup>-3'-cyanoethyl hydrogenophosphonate (δ 8.69, 8.64; 18.5%).

**Sulfurization:** To a solution of 5'-O-DMTr-dA<sup>Bz</sup>-dA<sup>Bz</sup>-3'-O-TBDMS phosphite triester dimer (0.2 mmol) in anhydrous acetonitrile is added AMBERLYST A26 tetrathionate form (5.4 eq., 1.14 mmol S<sub>4</sub>O<sub>6</sub><sup>2-</sup>, 0.63 g). The reaction mixture is shaken for 20 h. The reaction is followed by <sup>31</sup>P NMR. The yield is determined by <sup>31</sup>P NMR. After filtration of the resins the desired dimer dA-dA phosphorothioate triester is obtained with 88% of yield compared to the 5'-OH-

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dA<sup>Bz</sup>-3'-O-TBDMS. The crude is a mixture of 5'-O-DMTr-dA<sup>Bz</sup>-3'-cyanoethyl thiophosphoramidate (<sup>31</sup>P NMR (CD<sub>3</sub>CN) d 71.85, 71.22; 29.0%), 5'-O-DMTr-dA<sup>Bz</sup>-dA<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphorothioate (d 68.08, 68.01; 51.5%), 5'-O-DMTr-dA<sup>Bz</sup>-3'-cyanoethyl hydrogenophosphonate (d 8.66, 8.59; 19.5%).

## 5 Example 11

*Synthesis of the dimer 5'-OH-dC<sup>Bz</sup>-dA<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphorothioate triester.*

**Coupling procedure** of 5'-O-DMTr-dC<sup>Bz</sup>-3'-cyanoethyl-phosphoramidite with 5'-OH-dA<sup>Bz</sup>-3'-O-TBDMS using the poly(4-vinylpyridinium *p*-toluenesulfonate) (Aldrich):

5'-OH-dA<sup>Bz</sup>-3'-O-TBDMS adenosine (100 mg, 0.21 mmol) and 5'-O-DMTr-dC<sup>Bz</sup>-3'-cyanoethyl-phosphoramidite (365 mg, 0.43 mmol, 2. eq) are dissolved in anhydrous acetonitrile (15 ml). The solution is transferred under argon in a flask containing the poly(4-vinylpyridinium *p*-toluenesulfonate) (655 mg, 2.17 mmol tos<sup>-</sup>, 10.2 eq) and is shaken for 5 h 50 min. The reaction is followed by <sup>31</sup>P NMR. The yield is determined by <sup>31</sup>P NMR. The desired dC-dA phosphite triester dimer is obtained with 100% of yield compared to the 5'-OH-dA<sup>Bz</sup>-3'-O-TBDMS. The crude is a mixture of 5'-O-DMTr-dC<sup>Bz</sup>-dA<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphite triester (<sup>31</sup>P NMR (CD<sub>3</sub>CN) (d 140.55, 140.49; 53.9%), 5'-O-DMTr-dC<sup>Bz</sup>-3'-cyanoethyl hydrogenophosphonate (d 8.67; 18.5%).

**Sulfurization:** To a solution of 5'-O-DMTr-dC<sup>Bz</sup>-dA<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphite triester dimer (0.21 mmol) in anhydrous dichloromethane is added AMBERLYST A26 tetrathionate form (0.63 g, 1.14 mmol S<sub>4</sub>O<sub>6</sub><sup>2-</sup>, 5.3 eq.). The reaction mixture is shaken for 14 h 30 min. The reaction is followed by <sup>31</sup>P NMR. The yield is determined by <sup>31</sup>P NMR. After filtration of the resins the desired dC-dA phosphorothioate triester dimer is obtained with 100% of yield compared to the 5'-OH-dA<sup>Bz</sup>-3'-O-TBDMS. The crude is a mixture of 5'-O-DMTr-dC<sup>Bz</sup>-dA<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphorothioate (<sup>31</sup>P NMR (CD<sub>3</sub>CN) (d 68.14, 68.07; 50.6%), 5'-O-DMTr-dC<sup>Bz</sup>-3'-cyanoethyl hydrogenophos-

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phonate (d 8.67; 49.4%). Purification is attempted on a silica gel column, which is treated with triethylamine. Chromatography leads to complete loss of the cyanoethyl group. The dC<sup>Bz</sup>-dA<sup>Bz</sup> phosphorothioate dimer is eluted with CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH (80/1). The appropriate fractions are collected and evaporated to give a colorless oil. Yield: 185 mg, 0.14 mmol, 68%; <sup>31</sup>P NMR (CD<sub>3</sub>CN) d 57.58, 57.45; MALDI-TOF MS (positive mode, trihydroxyacetophenone as matrix) [M-DMTr+2H]<sup>+</sup> m/z<sub>exp</sub> = 879.42, m/z<sub>calc</sub> = 878.97.

**Example 12**

*Synthesis of the dimer 5'-OH-dA<sup>Bz</sup>-dA<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphorothioate triester.*

**Coupling procedure** of 5'-O-DMTr-dA<sup>Bz</sup>-3'-cyanoethyl-phosphoramidite with 5'-OH-dA<sup>Bz</sup>-3'-O-TBDMS using the poly(4-vinylpyridinium *p*-toluenesulfonate) (Aldrich):

5'-OH-dA<sup>Bz</sup>-3'-O-TBDMS (102 mg, 0.22 mmol) and 5'-O-DMTr-dA<sup>Bz</sup>-3'-cyanoethyl-phosphoramidite (381 mg, 0.44 mmol, 2.05 eq) are dissolved in anhydrous dichloromethane (15 ml). The solution is transferred under argon in a flask containing the poly(4-vinylpyridinium *p*-toluenesulfonate) (655 mg, 2.19 mmol tos<sup>-</sup>, 10.1 eq) and is shaken for 5 h 40 min. The reaction is followed by <sup>31</sup>P NMR. The yield is determined by <sup>31</sup>P NMR. The desired dA-dA phosphite triester dimer is obtained with 100% of yield compared to the 5'-OH-dA<sup>Bz</sup>-3'-O-TBDMS. The crude is a mixture of 5'-O-DMTr-dA<sup>Bz</sup>-dA<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphite triester (<sup>31</sup>P NMR (CD<sub>3</sub>CN) (d 140.77, 140.46; 66.9%), 5'-O-DMTr-dA<sup>Bz</sup>-3'-cyanoethyl hydrogenophosphonate (d 8.50, 8.41; 33.1%).

**Sulfurization:** To a solution of 5'-O-DMTr-dA<sup>Bz</sup>-dA<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphite triester dimer (0.22 mmol) in anhydrous dichloromethane is added AMBERLYST A26 tetrathionate form (0.87 g, 1.14 mmol S<sub>4</sub>O<sub>6</sub><sup>2-</sup>, 5.4 eq.). The reaction mixture is shaken for 22 h. The reaction is followed by <sup>31</sup>P NMR. The yield is determined by <sup>31</sup>P NMR. After filtration of the resins the desired dA-dA phosphorothioate triester dimer is obtained with 100 % of yield compared to



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the 5'-OH-A<sup>Bz</sup>-3'-O-TBDMS. The crude is a mixture of 5'-O-DMTr-dA<sup>Bz</sup>-dA<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphorothioate (<sup>31</sup>P NMR (CD<sub>3</sub>CN) (d 68.17, 67.89; 62.3%), 5'-O-DMTr-dA<sup>Bz</sup>-3'-cyanoethyl hydrogenophosphonate (d 8.45, 8.35; 37.7%).

- 5 **Detritylation:** To a solution of 5'-O-DMTr-dA<sup>Bz</sup>-dA<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphorothioate triester (0.22 mmol) in 10 ml CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH (7/3) is added 0.63 ml (0.3 mmol, 1.4 eq.) of a solution of benzene sulfonic acid 10% in CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH (7/3). The solution is stirred 45 min at 0°C. The reaction is washed with 10 ml of a saturated solution of NaHCO<sub>3</sub>, the organic layer is
- 10 separated, dried (Na<sub>2</sub>SO<sub>4</sub>), evaporated, and purified on a silica gel column. The desired dA-dA dimer is eluted with CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH (33/1). The appropriate fractions are collected and evaporated to give a colorless oil. Yield: 73 mg, 76 mmol, 35%; <sup>31</sup>P NMR (CD<sub>3</sub>CN) d 67.80, 67.71; MALDI-TOF MS (positive mode, trihydroxyacetophenone as matrix) [M-H]<sup>+</sup> m/z<sub>exp</sub> = 957.01, m/z<sub>calc</sub> = 957.07;
- 15 HPLC (spectrophotometrical purity at 260 nm = 95 %).

### Example 13

*Synthesis of the dimer 5'-OH-dG<sup>IBu</sup>-dA<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphorothioate triester.*

- 20 **Coupling procedure** of 5'-O-DMTr-dG<sup>IBu</sup>-3'-cyanoethyl-phosphoramidite with 5'-OH-dA<sup>Bz</sup>-3'-O-TBDMS using the poly(4-vinylpyridinium *p*-toluenesulfonate) (Aldrich):

- 5'-OH-dA-3'-O-TBDMS (100 mg, 0.21 mmol) and 5'-O-DMTr-dG<sup>IBu</sup>-3'-cyanoethyl-phosphoramidite (352 mg, 0.42 mmol, 1.97 eq) are dissolved in anhydrous acetonitrile (20 ml). The solution is transferred under argon in a
- 25 flask containing the poly(4-vinylpyridinium *p*-toluenesulfonate) (655 mg, 2.19 mmol tos<sup>-</sup>, 10.3 eq) and is shaken for 5 h 30 min. The reaction is followed by <sup>31</sup>P NMR. The yield is determined by <sup>31</sup>P NMR. The desired dG-dA phosphite triester dimer is obtained with 100% of yield compared to the 5'-OH-dA<sup>Bz</sup>-3'-O-TBDMS. The crude is a mixture of 5'-O-DMTr-dG<sup>IBu</sup>-dA<sup>Bz</sup>-3'-O-TBDMS cyano-

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ethyl phosphite triester ( $^{31}\text{P}$  NMR ( $\text{CD}_3\text{CN}$ ) ( $\delta$  140.65, 140.45; 51.2%), 5'-O-DMTr-dG<sup>IBu</sup>-3'-cyanoethyl hydrogenophosphonate ( $\delta$  9.00, 8.81; 48.8%).

**Sulfurization:** To a solution of 5'-O-DMTr-dG<sup>IBu</sup>-dA<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphite triester dimer (0.21 mmol) in anhydrous acetonitrile is added  
5 AMBERLYST A26 tetrathionate form (0.63 g, 1.14 mmol  $\text{S}_4\text{O}_6^{2-}$ , 5.4 eq.). The reaction mixture is shaken for 2 h. The reaction is followed by  $^{31}\text{P}$  NMR. The yield is determined by  $^{31}\text{P}$  NMR. After filtration of the resins the desired dG-dA phosphorothioate triester dimer is obtained with 100% of yield compared to the 5'-OH-dA<sup>Bz</sup>-3'-O-TBDMS. The crude is a mixture of 5'-O-DMTr-dG<sup>IBu</sup>-dA<sup>Bz</sup>-  
10 3'-O-TBDMS cyanoethyl phosphorothioate ( $^{31}\text{P}$  NMR ( $\text{CD}_3\text{CN}$ ) ( $\delta$  68.15, 68.02; 50.4%), 5'-O-DMTr-dG<sup>IBu</sup>-3'-cyanoethyl hydrogenophosphonate ( $\delta$  8.91, 8.68; 49.6%).

**Detritylation:** To a solution of 5'-O-DMTr-dG<sup>IBu</sup>-dA<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphorothioate triester (0.21 mmol) in 10 ml  $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$  (7/3) is added  
15 0.5 ml (0.3 mmol, 1.4 eq.) of a solution of benzene sulfonic acid 10% in  $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$  (7/3). The solution is stirred 20 min at 0°C. The reaction is washed with 10 ml of a saturated solution of  $\text{NaHCO}_3$ , the organic layer is separated, dried ( $\text{Na}_2\text{SO}_4$ ), evaporated, and purified on a silica gel column. The desired G-A dimer is eluted with  $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$  (33/1). The appropriate fractions are collected and evaporated to give a white foam. Yield: 95 mg, 0.1  
20 mmol, 48% with respect of 5'-OH-dA-3'-O-TBDM;  $^{31}\text{P}$  NMR ( $\text{CD}_3\text{CN}$ )  $\delta$  68.10, 67.87; HPLC (spectrophotometrical purity at 260 nm = 80%).

#### Example 14

*Synthesis of the dimer 5'-OH-dG<sup>IBu</sup>-dC<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphorothioate triester.*  
25

**Coupling procedure** of 5'-O-DMTr-dG<sup>IBu</sup>-3'-cyanoethyl-phosphoramidite with 5'-OH-dC<sup>Bz</sup>-3'-O-TBDMS using the poly(4-vinylpyridinium *p*-toluenesulfonate) (Aldrich):

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5'-OH-dC<sup>Bz</sup>-3'-O-TBDMS (100 mg, 0.22 mmol) and 5'-O-DMTr-dG<sup>IBu</sup>-3'-cyanoethyl-phosphoramidite (371 mg, 0.45 mmol, 2 eq) are dissolved in anhydrous acetonitrile (15 ml). The solution is transferred under argon in a flask containing the poly(4-vinylpyridinium *p*-toluenesulfonate) (690 mg, 2.3 mmol  
5 tos<sup>-</sup>, 10.3 eq) and is shaken for 5 h. The reaction is followed by <sup>31</sup>P NMR. The yield is determined by <sup>31</sup>P NMR. The desired dG-dC phosphite triester dimer is obtained with 100% of yield compared to the 5'-OH-dC<sup>Bz</sup>-3'-O-TBDMS. The crude is a mixture of 5'-O-DMTr-dG<sup>IBu</sup>-dC<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphite triester (<sup>31</sup>P NMR (CD<sub>3</sub>CN) (d 141.73, 141.26; 62.1%), 5'-O-DMTr-dG<sup>IBu</sup>-3'-  
10 cyanoethyl hydrogenophosphonate (d 9.05, 8.88; 37.9%).

**Sulfurization:** To a solution of 5'-O-DMTr-dG<sup>IBu</sup>-dC<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphite triester dimer (0.22 mmol) in anhydrous acetonitrile is added AMBERLYST A26 tetrathionate form (0.65 g, 1.3 mmol S<sub>4</sub>O<sub>6</sub><sup>2-</sup>, 5.4 eq.). The  
15 reaction mixture is shaken for 2 h. The reaction is followed by <sup>31</sup>P NMR. The yield is determined by <sup>31</sup>P NMR. After filtration of the resins the desired dG-dC phosphorothioate triester dimer is obtained with 100% of yield compared to the 5'-OH-dC<sup>Bz</sup>-3'-O-TBDMS. The crude is a mixture of 5'-O-DMTr-dG<sup>IBu</sup>-dC<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphorothioate (<sup>31</sup>P NMR (CD<sub>3</sub>CN) (d 68.12, 67.73; 61.2%), 5'-O-DMTr-dG<sup>IBu</sup>-3'-cyanoethyl phosphorothioate diester (d 56.51,  
20 56.39; 25.4%), 5'-O-DMTr-dG<sup>IBu</sup>-3'-cyanoethyl hydrogenophosphonate (d 9.04, 8.85; 25.4%). <sup>31</sup>P NMR d.

**Detritylation:** To a solution of 5'-O-DMTr-dG<sup>IBu</sup>-dC<sup>Bz</sup>-3'-O-TBDMS cyanoethyl phosphorothioate triester (0.22 mmol) in 10 ml CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH (7/3) is added 0.5 ml (0.3 mmol, 1.4 eq.) of a solution of benzene sulfonic acid 10% in  
25 CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH (7/3). The solution is stirred 1 h at 0°C. The reaction is washed with 10 ml of a saturated solution of NaHCO<sub>3</sub>, the organic layer is separated, dried (Na<sub>2</sub>SO<sub>4</sub>) and evaporated. The crude product is purified on a silica gel column using CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH (33:1). The appropriate fractions are collected and evaporated to give a colorless oil. Yield: 99 mg, 0.1 mmol, 47%; <sup>31</sup>P NMR  
30 (CD<sub>3</sub>CN) d 67.82, 67.56; MALDI-TOF MS (positive mode, trihydroxyacetophe-

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none as matrix)  $[M-H]^+$   $m/z_{\text{exp}} = 914.78$ ,  $m/z_{\text{calc}} = 914.03$ ; HPLC (spectrophotometrical purity at 260 nm = 84%).

**Example 15**

*Synthesis of 5'-O-DMTr-T-T-3'-O-DMTr-phosphorothioate diester.*

- 5 To a solution of 5'-O-DMTr-T-T-3'-O-DMTr *H*-phosphonate diester (25 mg, 22 mmol) in dichloromethane is added AMBERLYST A26 tetrathionate form (170 mg, 0.29 mmol  $S_4O_6^{2-}$ , 13 eq.) and 0.1 mL triethylamine. The reaction mixture is shaken for 78 h. The title compound was isolated after filtration of the resin and evaporation of the solvent. Yield: 28 mg, 22 mmol, 100%;  $^{31}\text{P}$  NMR (CD<sub>3</sub>CN)  $\delta$  57.22; MALDI-TOF MS (negative mode, trihydroxy-acetophenone as matrix)  $[M-H]^-$   $m/z_{\text{exp}} = 1166.23$ ,  $m/z_{\text{calc}} = 1666.25$ .

**Example 16**

*Synthesis of 5'-O-DMTr-T-T-3'-O-TBDMS-phosphorothioate diester.*

- 15 To a solution of 5'-O-DMTr-T-T-3'-O-TBDMS *H*-phosphonate (55 mg, 58 mmol) in dichloromethane is added AMBERLYST A26 tetrathionate form (250 mg, 0.42 mmol  $S_4O_6^{2-}$ , 7.3 eq.) and 0.2 mL triethylamine. The reaction mixture is shaken for 26 h. The title compound was isolated after filtration of the resin and evaporation of the solvent. Yield: 63 mg, 58 mmol, 100%;  $^{31}\text{P}$  NMR (CD<sub>3</sub>CN)  $\delta$  57.78, 57.72; MALDI-TOF MS (negative mode, trihydroxyacetophenone as matrix)  $[M-H]^-$   $m/z_{\text{exp}} = 977.04$ ,  $m/z_{\text{calc}} = 977.13$ .

**Example 17**

*Synthesis of AMBERLYST A26 tetrathionate form.*

- 25 10 g commercial Amberlyst A26 hydroxide form (Rohm & Haas) is washed twice with 20 mL methanol and twice with 20 mL dichloromethane and dried in vacuum. Potassium tetrathionate (30.35 g, 100 mmol, 3 eq.) is dissolved in 200 mL deionized water. The solution is added to the resin and shaken for 20 hours. The solution is decanted off. The resin is washed with 4 L deionized

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water, twice with 100 mL methanol and twice with 100 mL dichloromethane and dried under reduced pressure for 3 hours to give 8.5 g of solid-supported tetrathionate. The reagents loading was determined by elemental analysis, giving a value of 23.25% for sulfur (4.24% for nitrogen, 45.74% for carbon and less than 100 ppm for potassium). Loading: 1.81 mmol  $S_4O_6^{2-}$  per gram of resin.

**Example 18***Synthesis of polymer-supported pyridinium*

The commercially available strongly acidic ion-exchange resin DOWEX 50W X8  $H^+$  form (Fluka) is washed successively with water, HCl 2M, water until pH 7, methanol and dichloromethane to dry the resin. Then, the resin is stirred in a solution of pyridine 2M in acetonitrile or just washed with a slight flow of the solution of pyridine 2M in acetonitrile for 15 minutes. Then, the resin is washed with acetonitrile and dichloromethane and dried under vacuum over  $P_2O_5$ . The reagents loading was determined by elemental analysis, giving a value of 11.56% for sulfur and 3.97% for nitrogen. Loading: 2.83 mmol  $pyrH^+$  per gram of resin.

**Example 19***Preparation of polystyrene-bound acid chloride*

The commercial polystyrene-bound carboxy acid RAPP Polymere (5.0 g, 1.96 mmol/g, 100-200 mesh, 1% DVB) is suspended in anhydrous  $CH_2Cl_2$  (80 ml) and N,N-dimethylformamide (0.3 ml). Thionyl chloride (1.8 ml, 3.5 eq) are added under stirring and the mixture is refluxed for 3h. The resin is filtered under argon and washed with dried  $CH_2Cl_2$  (100 ml), ether (100 ml) and dried under vacuum for 4h.

IR ( $cm^{-1}$ ): 1775 ( $C=O$ , Acid chloride)

Elemental analysis: Cl 7.43% (w/100g resin) (2.09 mmol/g)

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Chloride titration: 2.1 mmol/g

### Example 20

#### *Synthesis of 5'-O-DMTr-dA<sup>Bz</sup>-dC<sup>Bz</sup>-3'-O-Lev H-phosphonate*

5 A solution of 5'-O-DMTr-dA<sup>Bz</sup>-H-phosphonate TEA salt (123.4 mg, 0.150 mmol) and of 3'-O-Lev-dC<sup>Bz</sup> (53.7 mg, 0.125 mmol) in 2.0 ml of CH<sub>2</sub>Cl<sub>2</sub>/py (1:1) is added to polystyrene-bound acid chloride (388.8 mg, 2.1 mmol/g, 5.5 eq) that is suspended in 2.5 ml of the same solvent. The mixture is shaken for 1h at room temperature until the disappearance of the monomers. The reaction is  
10 monitored by reverse phase HPLC. The resin is filtered, washed with CH<sub>2</sub>Cl<sub>2</sub>. The pyridinium salt present in solution is removed by aqueous extraction and the aqueous phase is washed twice with CH<sub>2</sub>Cl<sub>2</sub>. The organic fractions are collected, dried over Na<sub>2</sub>SO<sub>4</sub>, the solvent is evaporated and the pyridine is eliminated by coevaporation with toluene. The isolated product was dried under  
15 vacuum. Yield 89%.

<sup>31</sup>P NMR (CD<sub>3</sub>CN) δ 10.03 ppm, 9.46 ppm.

MALDI-TOF MS (positive mode, trihydroxyacetophenone as matrix) [M+H]<sup>+</sup>  
m/z<sub>exp</sub> = 1134.13, m/z<sub>calc</sub> = 1133.51.

The spectrophotometrical purity determined by HPLC is 93%.

### 20 Example 21

#### *Synthesis of 5'-O-DMTr-dA<sup>Bz</sup>-T-3'-O-Lev H-phosphonate*

A solution of 5'-O-DMTr-dA<sup>Bz</sup>-H-phosphonate TEA salt (123.4 mg, 0.150 mmol) and of 3'-O-Lev-T (42.5 mg, 0.125 mmol) in 2.0 ml of CH<sub>2</sub>Cl<sub>2</sub>/py (1:1) is added to polystyrene-bound acid chloride (550.0 mg, 2.1 mmol/g, 7.7 eq) that  
25 is suspended in 5.0 ml of the same solvent. The mixture is shaken for 30 min at room temperature until the disappearance of the monomers. The reaction is

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monitored by reverse phase HPLC. The resin is filtered, washed with  $\text{CH}_2\text{Cl}_2$ . The pyridinium salt present in solution is removed by aqueous extraction and the aqueous phase is washed twice with  $\text{CH}_2\text{Cl}_2$ . The organic fractions are collected, dried over  $\text{Na}_2\text{SO}_4$ , the solvent is evaporated and the pyridine is eliminated by coevaporation with toluene. The isolated product is dried under vacuum. Yield 88.5%

$^{31}\text{P}$  NMR ( $\text{CD}_3\text{CN}$ )  $\delta$  10.02 ppm, 9.08 ppm.

MALDI-TOF MS (positive mode, trihydroxyacetophenone as matrix)  $[\text{M}+\text{H}]^+$   
 $m/z_{\text{exp}} = 1043.02$ ,  $m/z_{\text{calc}} = 1045.00$ .

10 The spectrophotometrical purity determined by HPLC is 98%.

### Example 22

#### *Synthesis of 5'-O-DMTr-T-dC<sup>Bz</sup> -3'-O-TBDMS H-phosphonate*

A solution of 5'-O-DMTr-T-H-phosphonate TEA salt (106.4 mg, 0.150 mmol) and of 3'-O-TBDMS-dC<sup>Bz</sup> (55.7 mg, 0.125 mmol) in 2.0 ml of  $\text{CH}_2\text{Cl}_2/\text{py}$  (1:1) is added to polystyrene-bound acid chloride (555.0 mg, 2.7 mmol/g, 10 eq) that is suspended in 5.0 ml of the same solvent. The mixture is shaken for 2h at room temperature until the disappearance of the monomers. The reaction is monitored by reverse phase HPLC. The resin is filtered, washed with  $\text{CH}_2\text{Cl}_2$ . The pyridinium salt present in solution is removed by aqueous extraction and the aqueous phase is washed twice with  $\text{CH}_2\text{Cl}_2$ . The organic fractions are collected, dried over  $\text{Na}_2\text{SO}_4$ , the solvent is evaporated and the pyridine is eliminated by coevaporation with toluene. The isolated product is dried under vacuum. Yield 77.5%.

$^{31}\text{P}$  NMR ( $\text{CD}_3\text{CN}$ )  $\delta$  10.50 ppm, 10.00 ppm.

25 MALDI-TOF MS (positive mode, trihydroxyacetophenone as matrix)  $[\text{M}+\text{H}]^+$   
 $m/z_{\text{exp}} = 1038.69$ ,  $m/z_{\text{calc}} = 1037.20$ .

The spectrophotometrical purity determined by HPLC is 94%.

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**Example 23***Synthesis of 5'-O-DMTr -T-dC<sup>Bz</sup>-3'-O-TBDMS phosphorothioate TEA salt*

A solution of 5'-O-DMTr -T-dC<sup>Bz</sup>-3'-O-TBDMS H-phosphonate (50 mg, 0.048 mmol) in 5.0 ml of CH<sub>2</sub>Cl<sub>2</sub> and 0.2 ml TEA is added to Amberlyst A26 tetra-  
5 thionate form (141.0 mg, 1.7 mmol/g, 5 eq). The mixture is shaken over night, the the resin is filtered and the solvent is evaporated. The product is dried under vacuum. Yield 100%.

<sup>31</sup>P NMR (CD<sub>2</sub>Cl<sub>2</sub>) δ 59.17 ppm, 58.99 ppm.

10 The spectrophotometrical purity determined by HPLC is 95%.

**Example 24***Synthesis of 5'-O-DMTr-dA<sup>Bz</sup>-T-3'-O-Lev phosphate TEA salt*

A solution of 5'-O-DMTr-dA<sup>Bz</sup>-T-3'-O-Lev H-phosphonate (90 mg, 0.0863 mmol) in 5.0 ml of CH<sub>2</sub>Cl<sub>2</sub> and 0.2 ml TEA is added to  
15 (polystyrimethyl)trimethylamonium metaperiodate (NOVABIOCHEM) (173.0 mg, 2.5 mmol/g, 5 eq). The mixture is shaken over night, the resin is filtered and the solvent is evaporated. The product is dried under vacuum. Yield 100%.

<sup>31</sup>P NMR (CD<sub>2</sub>Cl<sub>2</sub>) δ -1.37 ppm.

20 MALDI-TOF MS (positive mode, trihydroxyacetophenone as matrix) [M+H]<sup>+</sup>  
m/z<sub>exp</sub> = 1059.31, m/z<sub>calc</sub> = 1060.03.

The spectrophotometrical purity determined by HPLC is 87%.

**Example 25***Synthesis of 5'-O-DMTr-T-dA<sup>Bz</sup>-dC<sup>Bz</sup>-3'-O-Lev H-phosphonate*



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Detritylation of 5'-O-DMTr-dA<sup>Bz</sup>-dC<sup>Bz</sup>-3'-O-Lev H-phosphonate

The H-phosphonate dimer 5'-O-DMTr-dA<sup>Bz</sup>-dC<sup>Bz</sup>-3'-O-Lev (120 mg, 0,106 mmol) is dissolved in 4.0 ml of CH<sub>2</sub>Cl<sub>2</sub>/MeOH (7:3) and cooled in an ice bath. To this solution 1.0 ml of a solution of 10% BSA (benzene sulfonic acid) in CH<sub>2</sub>Cl<sub>2</sub>/MeOH (7:3) is added drop wise under stirring and the progress of the reaction is monitored by TLC. After 15 min the mixture is quenched with a solution of NaHCO<sub>3</sub>. The organic layer is washed with water to remove any trace of base, then it is dried over Na<sub>2</sub>SO<sub>4</sub> and the solvent is evaporated. The product is purified by precipitation from CH<sub>2</sub>Cl<sub>2</sub> with ether and dried under vacuum. Yield 88%.

MALDI-TOF MS (positive mode, trihydroxyacetophenone as matrix) [M+H]<sup>+</sup> m/z<sub>exp</sub> = 830.75, m/z<sub>calc</sub> = 831.75.

The spectrophotometrical purity determined by HPLC is 91%.

Coupling

A solution of 5'-O-DMTr-T-H-phosphonate TEA salt (93.8 mg, 0.132 mmol) and of 5'-OH-dA<sup>Bz</sup>-dC<sup>Bz</sup>-3'-O-Lev H-phosphonate (73.2 mg, 0.088 mmol) in 2.0 ml of CH<sub>2</sub>Cl<sub>2</sub>/py (1:1) is added to polystyrene-bound acid chloride (503.0 mg, 2.1 mmol/g, 8 eq) that is suspended in 4.0 ml of the same solvent. The mixture is shaken for 1h at room temperature until the disappearance of the monomers. The reaction is monitored by reverse phase HPLC. The resin is filtered, washed with CH<sub>2</sub>Cl<sub>2</sub>. The pyridinium salt present in solution is removed by aqueous extraction and the aqueous phase is washed twice with CH<sub>2</sub>Cl<sub>2</sub>. The organic fractions are collected, dried over Na<sub>2</sub>SO<sub>4</sub>, the solvent is evaporated and the pyridine is eliminated by coevaporation with toluene. The isolated product is dried under vacuum. Yield 82%.

<sup>31</sup>P NMR (CD<sub>2</sub>Cl<sub>2</sub>) δ 10.23, 10.09, 9.70, 9.68, 9.52, 9.30, 9.24, 9.19 ppm.

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MALDI-TOF MS (positive mode, trihydroxyacetophenone as matrix)  $[M+H]^+$   
 $m/z_{\text{exp}} = 1421.06$ ,  $m/z_{\text{calc}} = 1422.33$ .

The spectrophotometrical purity determined by HPLC is 82%.

### Example 26

#### 5 *Synthesis of 5'-O-DMTr-dA<sup>Bz</sup>-dA<sup>Bz</sup>-T-3'-O-Lev H-phosphonate*

##### Detritylation of 5'-O-DMTr-dA<sup>Bz</sup>-T-3'-O-Lev H-phosphonate

The H-phosphonate dimer 5'-O-DMTr-dA<sup>Bz</sup>-T-3'-O-Lev (105 mg, 0,100 mmol) is dissolved in 4.0 ml of CH<sub>2</sub>Cl<sub>2</sub>/MeOH (7:3) and cooled in an ice bath. 1.0 ml of a solution of 10% BSA in CH<sub>2</sub>Cl<sub>2</sub>/MeOH (7:3) is added drop wise under stirring and the progress of the reaction is monitored by TLC. After 15 min the mixture is quenched with a solution of NaHCO<sub>3</sub>. The organic layer is washed with water to remove any trace of base, then it is dried over Na<sub>2</sub>SO<sub>4</sub> and the solvent is evaporated. The product is purified by precipitation from CH<sub>2</sub>Cl<sub>2</sub> in ether and dried under vacuum. Yield 70%.

15 MALDI-TOF MS (positive mode, trihydroxyacetophenone as matrix)  $[M+H]^+$   
 $m/z_{\text{exp}} = 742.25$ ,  $m/z_{\text{calc}} = 742.66$ .

The spectrophotometrical purity determined by HPLC is 92%.

##### Coupling

A solution of 5'-O-DMTr-dA<sup>Bz</sup>-H-phosphonate TEA salt (69.8 mg, 0.084 mmol) and of 5'-OH-dA<sup>Bz</sup>-dT-3'-O-Lev H-phosphonate (52.2 mg, 0.070 mmol) in 2.0 ml of CH<sub>2</sub>Cl<sub>2</sub>/py (1:1) is added to polystyrene-bound acid chloride (311.0 mg, 2.1 mmol/g, 7.7 eq) that is suspended in 2.0 ml of the same solvent. The mixture is shaken for 3h at room temperature until the disappearance of the monomers. The reaction is monitored by reverse phase HPLC. The resin is filtered, washed with CH<sub>2</sub>Cl<sub>2</sub>. The pyridinium salt present in solution is removed by aqueous extraction and the aqueous phase is washed twice with CH<sub>2</sub>Cl<sub>2</sub>.

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The organic fractions are collected, dried over  $\text{Na}_2\text{SO}_4$ , the solvent is evaporated and the pyridine is eliminated by coevaporation with toluene. The isolated product is dried under vacuum. Yield 75%.

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$^{31}\text{P}$  NMR ( $\text{CD}_2\text{Cl}_2$ )  $\delta$  10.09, 9.39, 8.82, 8.76, 8.30, 7.56 ppm.

5 MALDI-TOF MS (positive mode, trihydroxyacetophenone as matrix)  $[\text{M}+\text{H}]^+$   
 $m/z_{\text{exp}} = 1445.60$ ,  $m/z_{\text{calc}} = 1447.40$ .

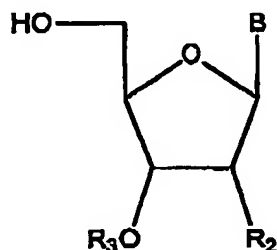
The spectrophotometrical purity determined by HPLC is 91.5%.

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**Claims**

1. A method for preparing an oligonucleotide comprising the steps of

a) providing a 3'-protected compound having the formula:



5        wherein

B is a heterocyclic base

R<sub>2</sub> is H, a protected 2'-hydroxyl group, F, a protected amino group, an O-alkyl group, an O-substituted alkyl, a substituted alkylamino or a C4'-O2' methylen linkage

10        R<sub>3</sub> is a hydroxyl protecting group, a 3'-protected nucleotide or a 3'-protected oligonucleotide

b) reacting said compound with a nucleotide derivative having a 5'-protection group in the presence of a solid supported activator to give an elongated oligonucleotide with a P(III)-internucleotide bond

15        c) processing the elongated oligonucleotide with a P(III)-Internucleotide bond by steps c1) and c2) in any sequence

c1) capping by reacting with a solid supported capping agent

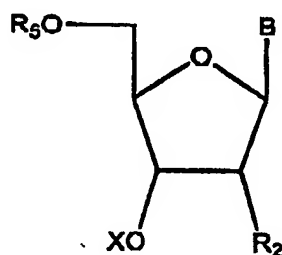
c2) oxidizing by reacting the oligonucleotide with a solid supported oxidizing reagent

20        d) removing the 5'-protection group by treatment with a solid supported agent or removing the 5'-protection group with a removal agent followed

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by addition of a solid supported scavenger or followed by extraction.

2. The method of claim 1, wherein the nucleotide derivative having a 5'-protection group of step b) has the following formula:



5 wherein

X is a P(III)-function

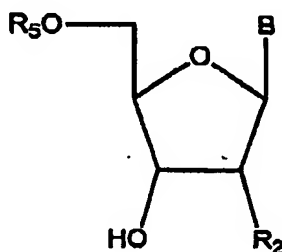
B is a heterocyclic base

R<sub>2</sub> is H, a protected 2'-hydroxyl group, F, a protected amino group, an O-alkyl group, an O-substituted alkyl, a substituted alkylamino or a C4'-O2' methylen linkage

R<sub>5</sub> is a hydroxyl protecting group, a 5'-protected nucleotide or a 5'-protected oligonucleotide.

3. A method for preparing an oligonucleotide comprising the steps of

a) providing a 5'-protected compound having the formula:



15 wherein

B is a heterocyclic base

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$R_2$  is H, a protected 2'-hydroxyl group, F, a protected amino group, an O-alkyl group, an O-substituted alkyl, a substituted alkylamino or a C4'-O2' methylen linkage

5  $R_3$  is a hydroxyl protecting group, a 5'-protected nucleotide or a 5'-protected oligonucleotide

b) reacting said compound with a nucleotide derivative having a 3'-protection group in the presence of a solid supported activator to give an elongated oligonucleotide with a P(III)-internucleotide bond

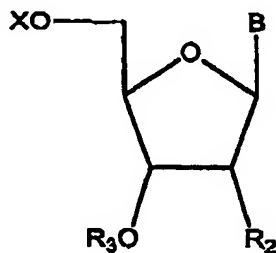
10 c) processing the elongated oligonucleotide with a P(III)-internucleotide bond by steps c1) and c2) in any sequence

c1) capping by reacting with a solid supported capping agent

c2) oxidizing by reacting the oligonucleotide with a solid supported oxidizing reagent

15 d) removing the 3'-protection group by treatment with a solid supported agent or removing the 3'-protection group with a removal agent followed by addition of a solid supported scavenger or followed by extraction.

4. The method of claim 3, wherein the nucleotide derivative having a 3'-protection group has the following formula:



20 wherein

X is a P(III)-function

B is a heterocyclic base

$R_2$  is H, a protected 2'-hydroxyl group, F, a protected amino group, an O-alkyl group, an O-substituted alkyl, a substituted alkylamino or a C4'-O2' methylen linkage

$R_5$  is a hydroxyl protecting group, a 5'-protected nucleotide or a 5'-protected oligonucleotide

5 5. The method of any one of claims 1 to 4, comprising the further step of e) repeating steps a) to d) at least once.

6. The method of any one of claims 1 to 5, wherein the nucleotide derivative of step b) is a phosphoramidite or a H-phosphonate.

10 7. The method of any one of steps 1 to 6, wherein the solid supported activator of step b) is selected from the group consisting of a solid support bearing a pyridinium salt, a cation exchange solid support with an optionally substituted pyridinium, or a cation exchange solid support with an optionally substituted imidazolium salt, a solid support bearing an optionally substituted  
15 azole (imidazol, triazole, tetrazole), a salt of a weak base anion exchange resin with a strong acid, a weak cation exchange resin (carboxylic) in its protonated form, a solid support bearing an optionally substituted phenol, a solid support bearing a carboxylic acid chloride/bromide, a sulfonic acid chloride/bromide, a chloroformate, a bromoformate, a chlorosulfite, a bromosul-  
20 fite, a phosphorochloridate and a phosphorbromidate.

8. The method of any one of claims 1 to 7, wherein the solid supported oxidizing reagent is selected from the group consisting of solid supported periodates, permanganates, osmium tetroxides, dichromates, hydroperoxides, substituted alkylamine oxides, percarboxylic acid and persulfonic acid.

25 9. The method of any one of claims 1 to 8, wherein the oxidizing is a sulfurization.

10. The method of claim 9, wherein the solid supported oxidizing reagent is selected from the group consisting of a solid supported tetrathionate, a solid

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supported alkyl or aryl sulfonyl disulfide, a solid supported optionally substituted dibenzoyl tetrasulfide, a solid supported bis(alkoxythio-carbonyl)tetrasulfide, a solid supported optionally substituted phenylacetyl disulfide, a solid supported N-[(alkyl or aryl)sulfanyl] alkyl or aryl substituted succinimide and a solid supported (2-pyridinyldithio) alkyl or aryl.

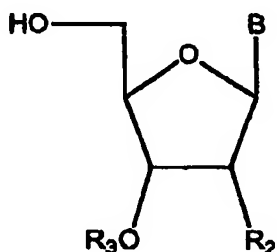
11. The method of any one of claims 1 to 10, wherein the solid supported capping agent is a solid supported activated acid, preferably a carboxylic acid chloride, carboxylic acid bromide, azolide, substituted azolide, anhydride or chloroformate or phosphorochloridate, or a solid supported phosphoramidite, or a solid supported H-phosphonate monoester.

12. The method of any one of claims 1 to 11, wherein the 5'-protection is a dimethoxytrityl group (DMTr) or a monomethoxytrityl group (MMTr) and the solid supported agent of step d) is an cationic ion exchanger resin in the H<sup>+</sup> form or ceric ammonium nitrate.

13. The method of any one of claims 1 to 12, wherein the 3'-protection is a silyl group and the solid supported agent of step d) is an anionic ion exchanger resin in the F-form.

14. Solid supported sulfurization agent consisting of solid supported amine and a tetrathionate having the formula S<sub>4</sub>O<sub>6</sub>.

15. A method for coupling a compound having the formula



wherein

B is a heterocyclic base



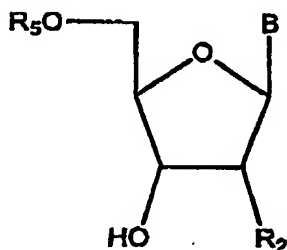
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$R_2$  is H, a protected 2'-hydroxyl group, F, a protected amino group, an O-alkyl group, an O-substituted alkyl, a substituted alkylamino or a C4'-O2'-methylen linkage

$R_3$  is a hydroxyl protecting group, a nucleotide or an oligonucleotide

- 5 with a nucleotide derivative having a 5'-protection group in the presence of a solid supported activator to give an elongated oligonucleotide with a P(III)-internucleotide bond

16. A method for coupling a compound having the formula



- 10 wherein

B is a heterocyclic base

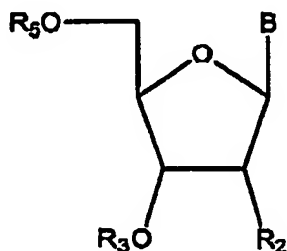
$R_2$  is H, a protected 2'-hydroxyl group, F, a protected amino group, an O-alkyl group, an O-substituted alkyl, a substituted alkylamino or a C4'-O2'-methylen linkage

- 15  $R_5$  is a hydroxyl protecting group, a 5'-protected nucleotide or a 5'-protected oligonucleotide

with a nucleotide derivative having a 3'-protection group in the presence of a solid supported activator to give an elongated oligonucleotide with a P(III)-internucleotide bond.

17. A method for preparing an oligonucleotide comprising the steps of

a) providing a compound having the formula:



wherein

5 B is a heterocyclic base

R<sub>2</sub> is H, a protected 2'-hydroxyl group, F, a protected amino group, an O-alkyl group, an O-substituted alkyl, a substituted alkylamino or a C4'-O2' methylen linkage

and

10 R<sub>3</sub> is a hydroxyl protecting group, a protected nucleotide or a protected oligonucleotide and R<sub>5</sub> is a P(III) function

or

R<sub>5</sub> is a hydroxyl protecting group, a protected nucleotide or a protected oligonucleotide and R<sub>3</sub> is a P(III) function

15 b) reacting said compound with a nucleotide derivative having a 3' or 5'-free OH-group in the presence of a solid supported activator to give an elongated oligonucleotide with a P(III)-internucleotide bond

c) processing the elongated oligonucleotide with a P(III)-internucleotide bond by steps c1) and c2) in any sequence

20 c1) capping by reacting with a solid supported capping agent

c2) oxidizing by reacting the oligonucleotide with a solid supported oxi-

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dizing reagent

d) removing the 3' or 5'-protection group by treatment with a solid supported agent or removing the 3' or 5'-protection group with a removal agent followed by addition of a solid supported scavenger or followed by extraction.

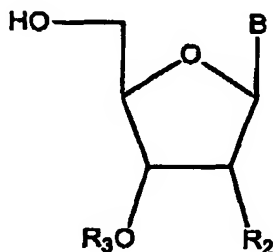
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# **Abstract**

A method for preparing an oligonucleotide comprising the steps of

a) providing a 3'-protected compound having the formula:



5 wherein

B is a heterocyclic base

R<sub>2</sub> is H, a protected 2'-hydroxyl group, F, a protected amino group, an O-alkyl group, an O-substituted alkyl, a substituted alkylamino or a C4'-O2' methylen linkage

10 R<sub>3</sub> is a hydroxyl protecting group, a 3'-protected nucleotide or a 3'-protected oligonucleotide

b) reacting said compound with a nucleotide derivative having a 5'-protection group in the presence of a solid supported activator to give an elongated oligonucleotide with a P(III)-internucleotide bond

15 c) processing the elongated oligonucleotide with a P(III)-internucleotide bond by steps c1) and c2) in any sequence

c1) capping by reacting with a solid supported capping agent

c2) oxidizing by reacting the oligonucleotide with a solid supported oxidizing reagent

20 d) removing the 5'-protection group by treatment with a solid supported agent or removing the 5'-protection group with a removal agent followed by addition of a solid supported scavenger or followed by extraction.

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